Evaluation of a commercial ELISA for the specific detection of antibodies against *Besnoitia besnoiti*

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ABSTRACT

Bovine besnoitiosis is an economically important disease in cattle caused by the protozoan parasite *Besnoitia besnoiti*, which occurs endemically in many countries of Africa and Asia and is spreading in Europe. Serological identification of subclinically infected cattle is important to avoid the introduction of infected animals into naive herds. Here we determine the sensitivity and specificity of the PrioCHECK® Besnoitia Ab, a serological test recently introduced into the European market. Analytical specificity was examined using sera from animals experimentally infected with parasites related to *B. besnoiti* ($n = 27$). Three animals experimentally infected with *Neospora caninum* or *Toxoplasma gondii* showed inconclusive reactions in the ELISA (percent positivity relative to the positive control [PP] $10\% \leq 20\%$) while all other sera reacted negative (PP < 10%). An estimate of the diagnostic specificity was obtained by analysing field sera from bovine herds without besnoitiosis but with abortion problems associated to *N. caninum* ($n = 403$). The analysis revealed a specificity of 94.3% or 96.8% depending on the applied cut-off (PP 10% or 20%, respectively). Sensitivity was assessed with sera from 110 animals of a herd in Germany where clinical bovine besnoitiosis was first diagnosed in September 2008. A positive serological reference standard was defined regarding sera from animals as reference positive, if these animals had tested positive in at least two of a panel of three other serological tests (two different *B. besnoiti*

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immunoblots and one immunofluorescence antibody test) on both of two sampling dates, November 2008 and April 2009. A diagnostic sensitivity of 91.8% or 75.5% was determined for sera collected in November 2008 and a sensitivity of 82.7% or 50% for sera collected in April 2009 (cut-off PP 10% or PP 20%, respectively). The marked drop in sensitivity from November 2008 to April 2009 was predominantly observed in reference-positive cattle without clinical signs. We conclude that PrioCHECK® Besnoitia Ab is a valuable diagnostic tool to detect clinically infected animals. Thus it may be used to support control measures, e.g., for the separation of infected animals from the remaining herd to avoid a further transmission of the infection within the herd.

1. Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan closely related to *Toxoplasma gondii* and *Neospora caninum*. It is the cause of bovine besnoitiosis, a severe but usually non-fatal disease with significant economic impact in many countries of Africa, Asia and Europe. Bovine besnoitiosis is re-emerging in Europe as recently reported by the European Food Safety Authority (http://www.efsa.europa.eu/en/scdocs/scdoc/1499.htm). The clinical hallmarks in acute infection are pyrexia, nasal and ocular discharge, salivation, stiff gait, and in severe cases subcutaneous oedema. Chronic infection in cattle is characterized by lichenified and alopecic skin; moreover, bulls may develop orchitis which may result in temporary or permanent infertility (Bigalke, 1968). Usually, only few cattle in an infected herd develop typical clinical signs while most animals remain subclinically infected (Bigalke, 1968). There is evidence that the disease has spread from southern to northern European regions (Alzieu et al., 2007), possibly due to animal trade but perhaps also facilitated by climatic changes (Kutz et al., 2009). Recently, confirmed cases of bovine besnoitiosis were reported in Germany (Mehlhorn et al., 2009; Schares et al., 2009; Majzoub et al., 2010; Rostaher et al., 2010). This outbreak showed that *B. besnoiti* infections can establish also in European countries north of the Alps.

B. besnoiti can be transmitted mechanically by tabanids and biting muscids (Bigalke, 1968). Its definitive host is not known (Diesing et al., 1988). Introduction of subclinically infected cattle into naive herds seems to play a major role in the transmission of the disease between herds, but also among countries (Bigalke, 1968). Therefore, sensitive and specific serological tests are urgently needed to detect subclinically infected cattle and to prevent their introduction into non-infected herds.

For the diagnosis of bovine *B. besnoiti* infection, a number of in-house serological techniques, including immunofluorescent antibody tests (IFAT), enzyme linked immunosorbent assays (ELISA), and immunoblots have been reported (Neuman, 1972; Shkap et al., 1984; Janitschke et al., 1984; Cortes et al., 2006a; Fernandez-Garcia et al., 2009; Schares et al., 2010). Recently, the PrioCHECK® Besnoitia Ab (Prionics AG, Schlieren, Switzerland) was introduced into the European market and a market authorisation for Germany has been granted in 2009. The aim of the present study was to determine the sensitivity and specificity of the PrioCHECK® Besnoitia Ab.

2. Material and methods

2.1. Sera

2.1.1. Sera from cattle experimentally infected with related protozoans

Sera from 27 cattle experimentally infected with *N. caninum* ($n = 2$; sera obtained on 0, 23, 57, 180 days post inoculation [dpi]), *T. gondii* ($n = 12$; 20–75 dpi), *Sarcocystis cruzi* ($n = 9$; 35 to 50 dpi), *Sarcocystis hominis* ($n = 2$; 43 dpi), or *Sarcocystis hirsuta* ($n = 2$; 34 dpi) (for details, see Schares et al., 1999) were included.

2.1.2. Sera from unaffected cattle

Sera of 403 cattle from 7 German herds with no history of bovine besnoitiosis were also used. These animals were sampled after *N. caninum*-associated abortions had been diagnosed by histology and PCR in these herds. All sera were examined for antibodies against *N. caninum* by methods previously published (Schares et al., 1998, 1999).

2.1.3. Sera from infected cattle

All sera used to evaluate the sensitivity of the ELISA were from cattle from a herd in Germany where clinical besnoitiosis was first diagnosed in September 2008 (Rostaher et al., 2010). All animals of this herd were clinically examined and sampled from a jugular or the tail vein in November 2008 and again in April 2009. Thereafter two different *B. besnoiti* immunoblots and a *B. besnoiti* IFAT were employed at a level of 100% specificity, as previously described (Schares et al., 2010). A serological reference standard was defined in which an animal was regarded as reference positive, if at least two of these three tests revealed a positive result for samples taken in both November 2008 and April 2009 (1 male, 109 females).

Animals with macroscopic tissue cysts in the scleral conjunctiva of one or both eyes or in the mucous membrane of the *vestibulum vaginae* were detected on both sampling dates. At the first sampling in November 2008, tissue samples and scrapings from the mucous membrane of the *vestibulum vaginae* from 29 animals with demonstrable tissues cysts were examined by histology and PCR (Schares et al., 2009).

2.2. Cell culture and purification of tachyzoites

The NC-1 strain of *N. caninum* (Dubey et al., 1988) and the Bb1Evora03 strain of *B. besnoiti* (Cortes et al., 2006b) were maintained in Vero cell cultures and purified as pre-

viously described (Schares et al., 1998, 1999). Purified tachyzoites were used to prepare IFAT slides or pelleted by centrifugation at $1300 \times g$ for 10 min and frozen at $-80\,°C$ until used for immunoblotting.

2.3. Isolation and purification of bradyzoites

Samples of lichenified and partially alopecic skin collected after slaughtering *B. besnoiti*-infected cattle from the first German bovine besnoitiosis case herd were frozen at $-20\,°C$ and submitted to the Friedrich–Loeffler-Institut, Wusterhausen, Germany. To remove surface contaminants, the external part of the skin sample was trimmed away and the core (1 g) was ground in Dulbecco's Modified Eagle Medium using a mortar and pestle. The parasites were purified with 3 g fibre glass wool (Sigma, Deisenhofen, Germany) filled into a 200 ml borosilicate glass burette. The homogenate (50 ml) was added to the glass wool column and washed with 200 ml phosphate buffered saline (PBS) until 80% of the parasites had passed the column. Eluted parasites were concentrated by centrifugation ($1300 \times g$; 10 min) and frozen at $-80\,°C$ until used for immunoblotting.

2.4. IFAT and immunoblot

IFAT and immunoblot were essentially performed as previously described (Schares et al., 2010). Briefly, suspensions of *B. besnoiti* tachyzoites (5×10^6 ml^{-1}) in PBS, pH 7.2, were air-dried on glass slides and frozen at $-20\,°C$ until used. The slides were fixed with ice-cold acetone prior to the examination. Sera were diluted in PBS. After serum incubation the slides were rinsed with fluorescent antibody (FA) buffer (25 mM Na_2CO_3, 100 mM $NaHCO_3$, 35 mM NaCl, pH 9.0) and PBS prior to conjugate incubation. Fluorescein isothiocyanate (FITC) conjugate (anti bovine IgG [H+L], Jackson Immuno Research Laboratories, West Grove, USA), diluted 1:50 in PBS with 0.05% Evans blue, was added and the slides examined with a fluorescence microscope (Olympus Vanox AHBT3, Hamburg, Germany). Only peripheral, but not apical fluorescence was considered specific. As previously shown, a cut-off titre of 200 in the IFAT resulted in a specificity of 100% and a sensitivity of 92% (Schares et al., 2010); a cut-off titre of 200 was therefore applied in the present study.

To prepare antigen-coated membranes by Western blotting samples containing 4×10^7 zoites were treated for 10 min at $94\,°C$ with non-reducing sample buffer (2% [w/v] sodium dodecyl sulfate [SDS], 10% [v/v] glycerol, 62 mM TrisHCl, pH 6.8) and electrophoresed in a SDS-polyacrylamide minigel. Separated parasite antigens and marker proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Germany) in a semi-dry transfer system (Pharmacia Biotech, Freiburg, Germany). The antigen-coated membrane was blocked with PBS-TG (PBS, 0.05% [v/v] Tween 20, 2% [v/v] fish gelatine liquid [Serva, Heidelberg, Germany]), air-dried overnight, cut into strips and stored frozen at $-20\,°C$ until used.

Prior to immunoblot analysis serum samples were diluted 1:200 in PBS-TG and the strips were blocked again with PBS-TG. After washing in PBS-T, (PBS, 0.05% [v/v] Tween 20), the strips were incubated with peroxidase conjugate solution (affinity purified goat anti-bovine IgG [H+L], Jackson ImmunoResearch Laboratories, West Grove, USA; diluted 1:500 in PBS-TG). After washing in PBS-T and distilled water, antibody reactions were detected by adding substrate solution (40 μl H_2O_2 [30% (v/v)] and 30 mg 4-chloro-1-naphthol [Sigma, Deisenhofen, Germany] in 40 ml PBS, 20% [v/v] methanol). Pre- and post-infection sera from a heifer experimentally infected with *N. caninum* (Schares et al., 1999) were used as negative controls and sera of three cattle naturally infected with *B. besnoiti* (Cortes et al., 2006a; Schares et al., 2009) as positive controls. In both the *B. besnoiti* tachyzoite and bradyzoite immunoblot, diagnosis was based on 10 selected specific bands (Schares et al., 2010). The presence of at least 4 of these specific bands was regarded as positive. With this cut-off, both immunoblot tests exhibited 100% specificity and 90% sensitivity (Schares et al., 2010).

2.5. ELISA

The PrioCHECK® Besnoitia Ab ELISA (Lot No. U 081103 M), which uses a conjugate specific for bovine IgG (PB, personal communication), was performed according to the manufacturer's instructions. Optical density (OD) was measured at 450 nm (reference filter 620 nm) and the test results were normalised and interpreted by calculating for each sample a percent positivity (PP) value relative to the OD of the positive control ($PP_{Sample} = OD_{450\,nm}$ sample/$OD_{450\,nm}$ positive control × 100). A $PP \geq 20\%$ was regarded as positive, PP values of 10–19% as inconclusive and a PP < 10% as negative.

2.6. Statistical methods

Sensitivity, specificity and test agreement (expressed as Kappa-values), were calculated using WinEpiscope 2.0 (N. de Blas et al: http://www.clive.ed.ac.uk/winepiscope/). Linear regression was performed using Statistica 7.1 (StatSoft. Inc., Tulsa, OK, USA).

3. Results

3.1. Analytical specificity of the PrioCHECK® Besnoitia Ab ELISA

The analytical specificity of the ELISA was determined using 33 sera obtained from 27 animals experimentally infected with *N. caninum*, *T. gondii*, *S. cruzi*, *S. hominis* or *S. hirsuta*. All but three sera revealed ELISA PP values of <10%. One serum, which originated from a *T. gondii*-infected animal, yielded a PP of 12.1%. Serially collected sera from an *N. caninum*-infected heifer had PP values of 15.6%, 19.6%, and 5.7%, at dpi 23, 57, and 180, respectively. Sera from another *N. caninum*-infected heifer showed PP values of 9.5%, 17.3% and 14.7%, at dpi 23, 57, and 180, respectively. The pre-infection sera of both heifers revealed ELISA PP values of <10%.

3.2. Diagnostic specificity of the PrioCHECK® Besnoitia Ab ELISA

To assess the diagnostic specificity of the ELISA, a set of sera ($n = 403$) from herds with *N. caninum*-associated abortion was used, which has previously been used to determine the diagnostic specificity of *B. besnoiti*-specific immunoblots and IFAT (Schares et al., 2010). Among these sera, 53.1% (214/403) yielded a positive *N. caninum* immunoblot result. When a cut-off of PP 20% was applied, the ELISA revealed a specificity of 96.8% (0.95 CI: 94.4–98.2%). An ELISA-positive result (cut-off: PP 20%) was associated with an antibody response positive for *N. caninum* ($P = 0.04$; Yates corrected Chi-Square). When a cut-off of PP 10% was applied, a specificity of 94.3% (0.95 CI: 91.4–96.3%) was obtained. With the lower cut-off of PP 10%, there was no statistically significant association of a positive antibody response against *N. caninum* and a positive ELISA result within the group of animals from herds without besnoitiosis ($P > 0.05$; Yates corrected Chi-Square).

The highest *B. besnoiti* IFAT titre of the sera of unaffected cattle used to assess the specificity of the ELISA was 50. Regardless of the cut-off used in the ELISA, there was no statistically significant association of a positive reaction in the ELISA and an IFAT titre of 50 ($P > 0.05$; Yates corrected Chi-Square).

The maximum number of bands recognized in the *B. besnoiti* tachyzoite and bradyzoite immunoblot by the sera used to test specificity was 3 or 4, respectively. All sera that detected 3 or 4 bands in the *B. besnoiti* tachyzoite or bradyzoite immunoblot had an ELISA PP of <10%. Sera with an ELISA PP $\geq$ 10% recognized up to 2 and sera with an ELISA PP $\geq$ 20% no or 1 band. There was no statistically significant association between reactions with tachyzoite- or bradyzoite-specific bands and a positive ELISA reaction, regardless of the PP value used as the cut-off ($P > 0.05$; Yates corrected Chi-Square).

3.3. Diagnostic sensitivity of the PrioCHECK® Besnoitia Ab ELISA

A total number of 110 sera collected from all reference-positive animals in November 2008 and in April 2009 were used. Out of the samples collected in November 2008, 101 (91.8%, 0.95 CI: 84.6–96.0%) tested positive using an ELISA PP of 10% as the cut-off. When an ELISA PP of 20% was applied as the positive cut-off, 83 (75.5%, 0.95 CI: 66.2–82.9%) of these 110 sera tested positive. The number of positive sera was reduced when sera collected about 5 months later in April 2009, from the same 110 animals were analysed. In April 2009, 91 (82.7%, 0.95 CI: 74.1–89.0%) or 55 (50.0%, 0.95 CI: 40.4–59.6%) out of these 110 reference-positive sera tested positive, using an ELISA PP of 10% or 20% as positive cut-off, respectively.

3.3.1. Reference-positive cattle with demonstrable tissue cysts

At both samplings, the entire herd had been examined for macroscopic tissue cysts in the scleral conjunctivae and in the mucous membrane of the *vestibulum vaginae* at clinical examinations. A proportion of 47.4% (52/110) of the reference-positive animals showed signs of clinical besnoitiosis on at least one of the two occasions.

When the analysis of the PrioCHECK® Besnoitia Ab ELISA was restricted to these animals, 47 (90.4%, 0.95 CI: 78.2–96.4%) or 41 sera (78.9%, 0.95 CI: 64.9–88.5%) tested positive using an ELISA PP of 10% or 20% as the positive cut-off, respectively, in sera collected in November 2008.

When reference-positive sera from these same 52 animals collected in April 2009 were analysed at a cut-off of PP 10%, 48 (92.3%, 0.95 CI: 80.6–97.5%) tested positive. However, with a PP of 20% as the positive cut-off, only 35 (67.3%, 0.95 CI: 52.8–79.3%) were positive.

The ELISA indices obtained for these 52 individual animals sampled in November 2008 and April 2009 were significantly ($P < 0.001$) correlated ($R^2 = 0.7201$) (Fig. 1A).

3.3.2. Reference-positive cattle with demonstrable tissue cysts, confirmed by PCR or histological examination

On the first sampling date, tissue samples and scrapings from the mucous membrane of the *vestibulum vaginae* were examined in animals with demonstrable tissue cysts. In 19 of these animals PCR or the histological examination revealed a positive result. In sera collected in November 2008, 18 (94.7%, 0.95 CI: 71.9–99.7%) or 17 (89.5%, 0.95 CI: 65.5–98.2%) of these 19 sera tested positive using an ELISA PP of 10% or 20% as the positive cut-off, respectively. When reference-positive animals sampled in April 2009 were analysed, 18 reacted positive (94.7%, 0.95 CI: 71.9–99.7%) regardless of the cut-off applied. The ELISA indices obtained for these 19 animals in November 2008 and April 2009 were significantly ($P < 0.001$) correlated ($R^2 = 0.6804$) (Fig. 1B).

3.3.3. Reference-positive cattle without demonstrable tissue cysts

This analysis of the PrioCHECK® Besnoitia Ab ELISA was restricted to those reference-positive animals that had no demonstrable tissue cysts in November 2008 or April 2009, but had tested reference-positive at both sampling dates ($n = 58$). Fifty-two (89.7%, 0.95 CI: 78.2–95.7%) or 38 (65.5%, 0.95 CI: 51.8–77.2%) of these sera tested positive using a PP 10% or 20%, respectively, as the positive cut-off in the samples obtained in November 2008.

However, when reference-positive sera from the same animals collected in April 2009 were analysed, only 43 (74.1%, 0.95 CI: 60.7–84.4%) or 20 (34.5%, 0.95 CI: 22.8–48.2%) tested positive, using a PP of 10% or 20%, respectively, as the positive cut-off. The ELISA indices obtained for most of the individual animals were higher in November 2008 than in April 2009.

The correlation of the index values of the paired serum samples obtained from the same animals in November 2008 and April 2009 was statistically significant ($P < 0.001$; $R^2 = 0.6103$) (Fig. 1C).

### 3.4. Comparison of the ELISA with other serological tests

3.4.1. Comparison of IFAT versus ELISA

For both sample subsets, obtained in November 2008 and April 2009, the ELISA PP values tended to increase with increasing IFAT titres (Fig. 2A and B). A linear regression
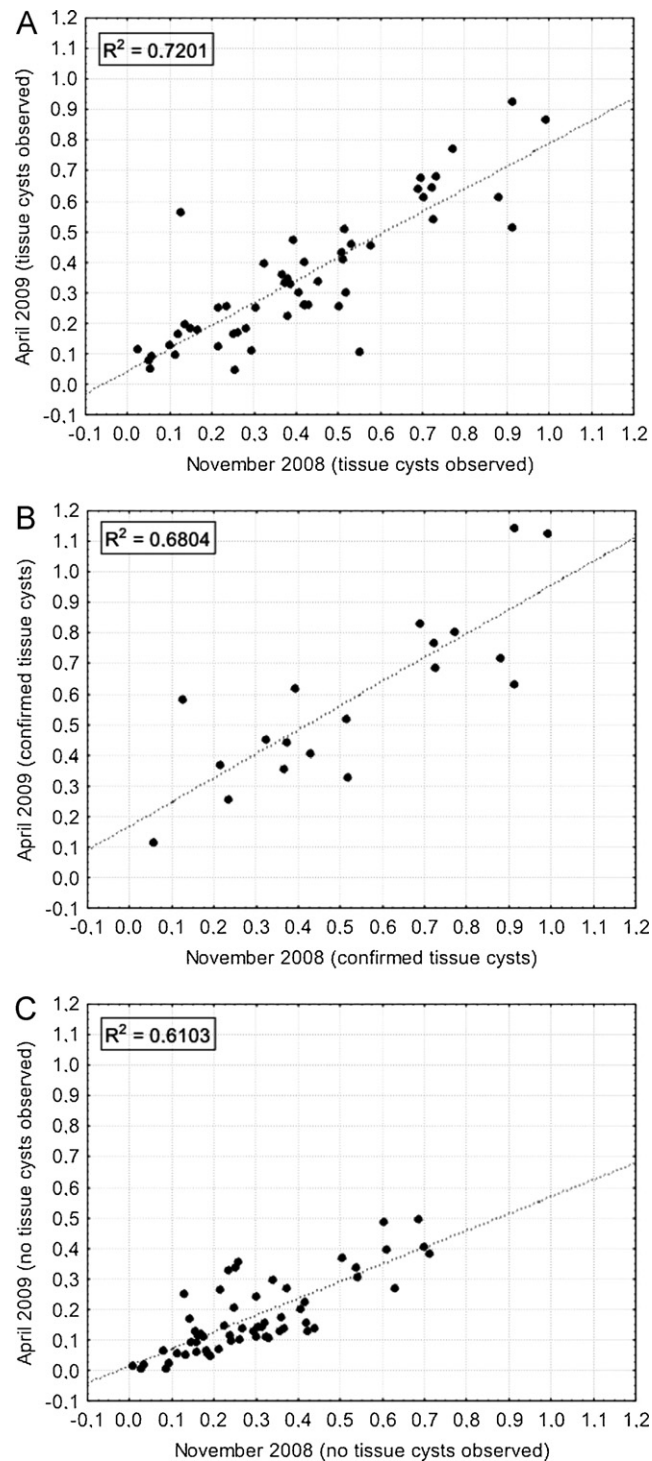
Fig. 1. Correlation of ELISA indices obtained for cattle in a herd with bovine besnoitiosis. The analysis was restricted to animals ($n = 110$) which had tested positive in November 2008 and in April 2009 in at least two of three serological tests (two different *B. besnoiti* immunoblots and one immunofluorescence antibody test). (A) Reference-positive animals which showed tissue cysts either in November 2008 or in April 2009 ($n = 52$). (B) Reference-positive animals which showed tissue cysts in November 2008 and had been confirmed as *Besnoitia besnoiti* infected by PCR or histological examination ($n = 19$). (C) Reference-positive animals which showed tissue cysts neither in November 2008 nor in April 2009 ($n = 52$). The dotted lines represent the result of linear regression analyses which revealed R^2 values of 0.7201, 06802 or 0.6103 in animals with clinically detectable tissue cysts, with clinically detectable tissue cysts confirmed by PCR and histology, and without clinically detectable tissue cysts, respectively. Note that the slope of the regression line in animals with no macroscopic tissue cysts (C) is lower than in the remaining (A and B).
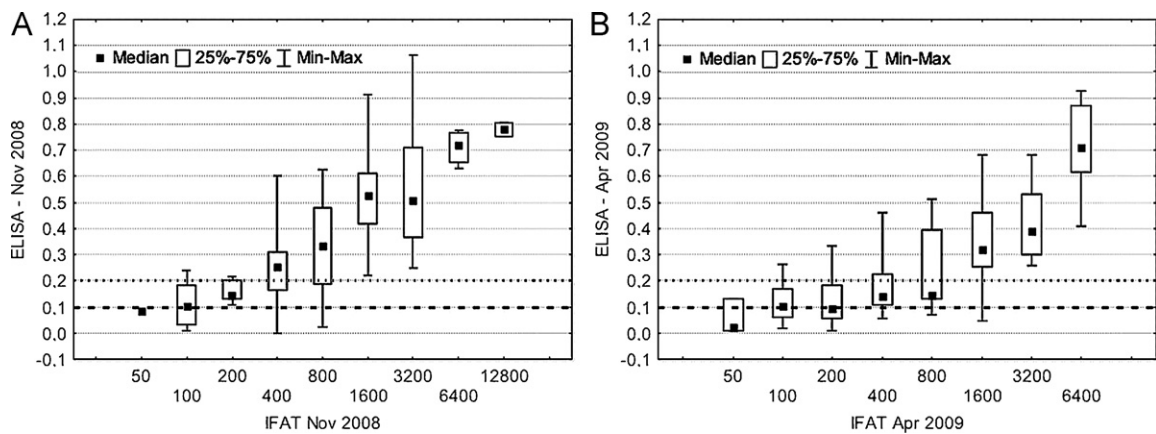
Fig. 2. Comparison of the titres in the immunofluorescent antibody test and the percent positivity (PP) values in the PrioCHECK Besnoitia Ab ELISA for 110 sera of reference-positive animals sampled in November 2008 (A) and in April 2009 (B). For the ELISA, the cut-offs at 10 PP (. . .) and 20 PP (–) are marked.

analysis performed with the ELISA and IFAT data revealed a statistically significant ($P < 0.001$) correlation with corrected R^2 of 0.34 or 0.52 for November 2008 or April 2009, respectively. Most animals (3/4 [75%]) with IFAT titres of 50 and less tested negative (cut-off: PP < 10%) (Table 1). Many (22/35 [63%]) sera with IFAT titres of 100 and 200 tested positive at a cut-off of PP 10%, but only few (7/35 [20%]) were positive at a cut-off of PP ≥ 20% (Fig. 2A and B). The majority (131/181 [72.4%] or 169/181 [93.4%]) of sera with IFAT titres of ≥400 tested positive in the ELISA at a cut-off of PP ≥ 20% or ≥10%, respectively (Table 1).

3.4.2. Comparison of B. besnoiti tachyzoite immunoblot versus ELISA

The ELISA PP values tended to increase with the number of bands recognized within a panel of 10 selected tachyzoite antigens (Fig. 3A and C). Linear regression analysis of the ELISA versus the tachyzoite immunoblot data revealed a statistically significant ($P < 0.001$) correlation with corrected R^2 values of 0.19 or 0.38 for the serum samples obtained in November 2008 or April 2009, respectively.

Most sera (126/157 [80%]) that recognized 7 bands or more had ELISA PP values ≥20% (Fig. 3A and C). Almost half of the sera (24/51 [47%]) recognizing 5 or 6 bands had ELISA PP values of ≥10% (Fig. 3A and C). Many (9/12 [75%]) samples recognizing only 4 bands had ELISA PP values < 10% (Fig. 3A and C).

3.4.3. Comparison of B. besnoiti bradyzoite immunoblot versus ELISA

The ELISA PP values tended to increase with the number of bands recognized within a panel of 10 selected bradyzoite antigens (Fig. 3B and D). Linear regression analysis of the ELISA versus the bradyzoite immunoblot data revealed a statistically significant ($P < 0.001$) correlation with corrected R^2 values of 0.13 or 0.18 for the serum samples obtained in November 2008 or April 2009, respectively.

Most sera (127/166 [77%]) that recognized 8 bands or more had ELISA PP values ≥20% (Fig. 3B and D). Many sera (32/42 [76%]) recognizing 6 or 7 bands had ELISA PP values of ≥10% (Fig. 3B and D). Almost half of the samples (5/11 [45%]) recognizing 4 or 5 bands, especially those from April 2009, had ELISA PP values < 10% (Fig. 3B and D).

4. Discussion

To control bovine besnoitiosis in afflicted herds or to avoid the introduction of infected animals into naive herds, highly sensitive and specific serological tests are required. Recently, the PrioCHECK® Besnoitia Ab was licensed in Germany and introduced to the European market.

According to the manufacturer's instructions, the PrioCHECK® Besnoitia Ab results are to be interpreted with two cut-offs. One is intended to identify infected cattle (ELISA PP ≥20%), while animals with ELISA PP values <10% are regarded as serologically negative and those with PP ≥10% and <20% are classified as inconclusive and should be retested.

Table 1
Comparison of titres in the immunofluorescent antibody test (IFAT) and the percent positivity (PP) values in the PrioCHECK® Besnoitia Ab ELISA for 220 sera from 110 reference positive animals sampled in November 2008 and April 2009.

ELISA	IFAT titre									Σ
	50	100	200	400	800	1600	3200	6400	12800	
<10 PP	3	6	7	8	3	1				28
10–19 PP	1	5	10	21	13	4				54
≥20 PP		2	5	26	21	45	27	10	2	138
Σ	4	13	22	55	37	50	27	10	2	220
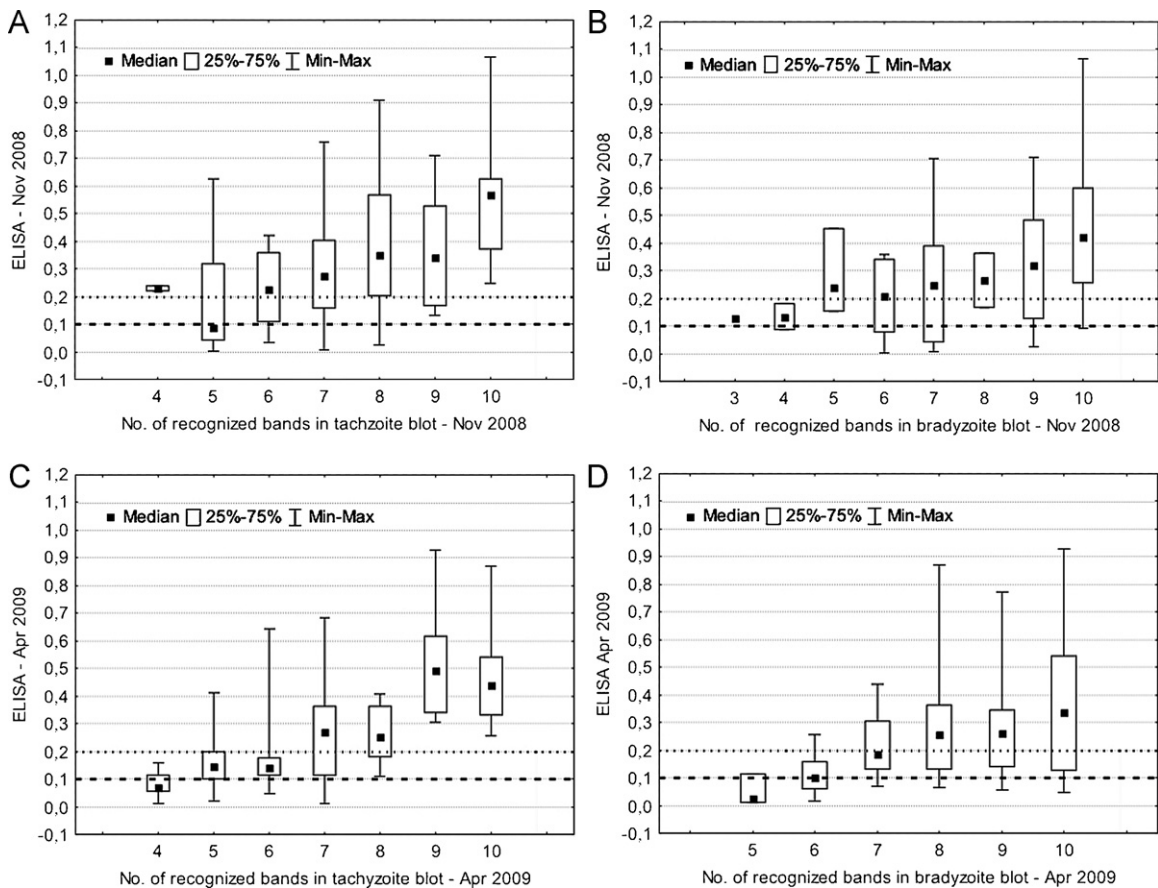
Fig. 3. Comparison of the reactions in the tachyzoite (A, C) and the bradyzoite (B, D) immunoblot and the percent positivity values in the PrioCHECK® Besnoitia Ab ELISA for 110 sera of reference-positive animals sampled in November 2008 (A, B) and in April 2009 (C, D). In the immunoblots, only positive reactions against ten selected tachyzoite antigens (45, 40, 37, 34, 30, 27, 22, 17, 16, 15 kDa) or against ten selected bradyzoite antigens (41, 36, 33, 28, 26, 24, 23, 22, 20, 18 kDa) were recorded (Schares et al., 2010). For the ELISA, the cut-offs at 10 PP (. . .) and 20 PP (−) are marked.

The specificity of the ELISA was assessed using sera from animals experimentally infected with related parasites but also field sera from bovine herds with *N. caninum*-associated abortion. We used samples from the latter herds since *N. caninum* is a protozoan closely related to *B. besnoiti* and because high *N. caninum* seroprevalences have been reported for European cattle populations (Bartels et al., 2006). Three of the 27 animals experimentally infected with *N. caninum* or *T. gondii* showed inconclusive reactions in the ELISA while all other sera reacted negative. Determination of the diagnostic specificity of the test with 403 field sera revealed also a statistically significant association of positivity in the ELISA with the presence of specific antibodies in the *N. caninum* immunoblot when the more stringent cut-off (≥20%) was applied to the PrioCHECK® Besnoitia Ab. These result suggest the presence of cross-reacting antigens in *B. besnoiti* and *N. caninum*, which is in accord with previous findings (Shkap et al., 2002; Schares et al., 2010).

To analyse the sensitivity of the PrioCHECK® Besnoitia Ab, we generated a set of positive sera from the first recorded outbreak of bovine besnoitiosis in Germany. We selected sera obtained in November 2008 and April

2009 from animals which had tested positive in other assays (IFAT, tachyzoite and bradyzoite immunoblot) on both sampling occasions. The sensitivity and specificity of these three serological tests have been recently described (Schares et al., 2010). To establish a reference set of positive sera for this study, we used these three tests at a level of 100% specificity to avoid including false positive sera.

For the reference-positive sera collected in November 2008, we observed a sensitivity of 75.5% and for those collected from the same animals in April 2009 a sensitivity of only 50%, when the more stringent cut-off (PP ≥20%) was applied in the PrioCHECK® Besnoitia Ab. When we added results originally considered as 'inconclusive' in the PrioCHECK® Besnoitia Ab to the positives by using a cut-off of PP ≥10%, the sensitivity was considerably increased to 91.8% for sera collected in November 2008 and to 82.7% for sera collected in April 2009 from the same group of animals. The marked drop in the ELISA sensitivity for the samples obtained in April 2009 from the reference-positive animals was predominantly caused by animals in which no tissue cysts had been found by clinical examination in November 2008 or April 2009. This may suggest that these animals were not boosted to a sufficient extent with

parasite antigens between November 2008 and April 2009 to maintain a detectable level of antibodies. Since the activity of putative arthropod vectors is low during winter months, our observation could be interpreted as an indication that the repeated transmission of *B. besnoiti* stages by mechanical transmission via blood sucking insects might have an impact on the level of antibodies against the parasite. In animals with a parasite load sufficient to cause tissue cysts detectable by clinical examination, the antigen load may have been sufficient to maintain high antibody levels until the next spring.

Within the group of animals with no demonstrable tissue cysts, the sensitivity of the PrioCHECK® Besnoitia Ab was low in April 2009 (74.1% or 34.5%, depending on the cut-off). However, since no tissue cysts were found in the scleral conjunctiva or in the *vestibulum vaginae,* it cannot be stated whether these animals were still infected with *B. besnoiti*. It is not yet known whether animals with no detectable tissue cysts in the scleral conjunctiva or in the mucous membranes of the *vestibulum vaginae* pose a risk to transmit the infection to other animals. The unclear epidemiological role of in-apparently or subclinically infected cattle and the fact that the PrioCHECK® Besnoitia Ab may not reliably detect such animals illustrates the need for further studies on the epidemiology of bovine besnoitiosis to elucidate the infection risk seropositive animals without detectable tissue cysts pose to others.

The present results clearly show that animals which tested 'inconclusive' by the PrioCHECK® Besnoitia Ab (i.e. animals with PP values of 10% to <20%) should be regarded as putatively infected. Since the diagnostic specificity was calculated as below 95% at the cut-off of PP 10%, positive results obtained in the PrioCHECK® Besnoitia Ab need to be verified by other serological tests. Lowering the cut-off of the PrioCHECK® Besnoitia Ab below PP 10% is unlikely to yield satisfactory results as the number of false positives may increase dramatically. Nevertheless, the PrioCHECK® Besnoitia Ab is a valuable diagnostic tool to detect animals with *B. besnoiti*-specific antibodies in bovine herds affected by besnoitiosis. The use of this ELISA could support control measures needed in outbreaks of bovine besnoitiosis, e.g. separation of the majority of infected animals from the remaining herd to avoid further spread of the disease within the herd.

Conflict of interest

The authors TH, MP, AR and PB are employees of Prionics AG, Switzerland. Their scientific contribution to this paper was the development of the ELISA. When the test was ready to use, it was passed over for validation to GS and FJC, Friedrich-Loeffler-Institute, Germany. Employees of Prionics AG had no influence on the planning and conduction of the validation experiments, data analysis and interpretation.

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References

Alzieu, J.-P., Dorchies, P., Schelcher, F., Gottstein, B., 2007. L'extension de la besnoitiose bovine en France. Le Point Vétérinaire 276, 37–43.

Bartels, C.J.M., Arnaiz-Seco, J.I., Ruiz-Santa-Quitera, A., Björkmann, C., Frössling, J., Von Blumröder, D., Conraths, F.J., Schares, G., van Maanen, C., Wouda, W., Ortega-Mora, L.M., 2006. Supranational comparison of *Neospora caninum* seroprevalences in cattle in Germany, The Netherlands, Spain and Sweden. Vet. Parasitol. 137, 17–27.

Bigalke, R.D., 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. Onderstepoort. J. Vet. Res. 35, 3–137.

Cortes, H.C., Nunes, S., Reis, Y., Staubli, D., Vidal, R., Sager, H., Leitao, A., Gottstein, B., 2006a. Immunodiagnosis of *Besnoitia besnoiti* infection by ELISA and Western blot. Vet. Parasitol. 141, 216–225.

Cortes, H.C., Reis, Y., Waap, H., Vidal, R., Soares, H., Marques, I., Pereira, d.F., Fazendeiro, I., Ferreira, M.L., Caeiro, V., Shkap, V., Hemphill, A., Leitao, A., 2006b. Isolation of *Besnoitia besnoiti* from infected cattle in Portugal. Vet. Parasitol. 141, 226–233.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.

Diesing, L., Heydorn, A.O., Matuschka, F.R., Bauer, C., Pipano, E., de Waal, D.T., Potgieter, F.T., 1988. *Besnoitia besnoiti*: studies on the definitive host and experimental infections in cattle. Parasitol. Res. 75, 114–117.

Fernandez-Garcia, A., Alvarez-Garcia, G., Risco-Castillo, V., Aguado-Martinez, A., Marugan-Hernandez, V., Ortega-Mora, L.M., 2009. Pattern of recognition of *Besnoitia besnoiti* tachyzoite and bradyzoite antigens by naturally infected cattle. Vet. Parasitol. 164, 104–110.

Janitschke, K., De Vos, A.J., Bigalke, R.D., 1984. Serodiagnosis of bovine besnoitiosis by ELISA and immunofluorescence tests. Onderstepoort J. Vet. Res. 51, 239–243.

Kutz, S.J., Jenkins, E.J., Veitch, A.M., Ducrocq, J., Polley, L., Elkin, B., Lair, S., 2009. The Arctic as a model for anticipating, preventing, and mitigating climate change impacts on host-parasite interactions. Vet. Parasitol. 163, 217–228.

Majzoub, M., Breuer, W., Gollnick, N.S., Rostaher, A., Schares, G., Hermanns, W., 2010. Ein Ausbruch von Besnoitiose bei Rindern in Deutschland: pathomorphologische, ultrastrukturelle und molekularbiologische Untersuchungen. Wien. Tierärztl. Mschr. 97, 9–15.

Mehlhorn, H., Klimpel, S., Schein, E., Heydorn, A.O., Al Quraishy, S., Selmair, J., 2009. Another african disease in central Europa: besnoitiosis of cattle. I. Light and electron microscopical study. Parasitol. Res. 104, 861–868.

Neuman, M., 1972. Serological survey of *Besnoitia besnoiti* (Marotel 1912) infection in Israel by immunofluorescence. Zbl. Vet. Med. B 19, 391–396.

Rostaher, A., Mueller, R.S., Majzoub, M., Schares, G., Gollnick, N.S., 2010. Bovine besnoitiosis in Germany. Vet. Dermatol. 21, 329–334.

Schares, G., Peters, M., Wurm, R., Bärwald, A., Conraths, F.J., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol. 80, 87–98.

Schares, G., Rauser, M., Zimmer, K., Peters, M., Wurm, R., Dubey, J.P., DeGraaf, D.C., Edelhofer, R., Mertens, C., Hess, G., Conraths, F.J., 1999. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. J. Parasitol. 85, 688–694.

Schares, G., Basso, W., Majzoub, M., Cortes, H.C., Rostaher, A., Selmair, J., Hermanns, W., Conraths, F.J., Gollnick, N.S., 2009. First in vitro isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet. Parasitol. 163, 315–322.

Schares, G., Basso, W., Majzoub, M., Rostaher, A., Scharr, J.-C., Langenmayer, J., Selmair, J., Dubey, J.P., Cortes, H.C., Conraths, F.J., Gollnick, N.S., 2010. Comparative evaluation of immunofluorescence antibody and novel immunoblot tests for the specific detection of bovine antibodies against *Besnoitia besnoiti* tachyzoites and bradyzoites. Vet. Parasitol. 171, 32–40.

Shkap, V., Ungar-Waron, H., Pipano, E., Greenblatt, C., 1984. Enzyme linked immunosorbent assay for detection of antibodies against *Besnoitia besnoiti* in cattle. Trop. Anim. Health Prod. 16, 233–238.

Shkap, V., Reske, A., Pipano, E., Fish, L., Baszler, T., 2002. Immunological relationship between *Neospora caninum* and *Besnoitia besnoiti*. Vet. Parasitol. 106, 35–43.